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Washington, DC 20231

Dated: September 5, 2000

Sir:

Transmitted herewith for filing is the utility patent application of:

Inventor(s): Lie-Fen SHYUR, Jui-Lin CHEN, Ning-Sun YANG

For: A Truncated Form of Fibrobacter Succinogenes 1,3-1,4-Beta-D-Glucanase With Improved Enzymatic Activity And Thermo-Tolerance

Enclosed are:

- Transmittal letter **(2x)** with Fee Computation Sheet
- General Authorization For Payment of Fees **(2x)**
- Title Page, Specification, Claims 1 to 16 & Abstract (25 pages [total number of pages of application])
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- 7 sheet(s) of drawing(s) (Figs. 1 to 6)
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[] Priority is claimed for this invention and application, corresponding applications having been filed in on , No. , on , No. , on , No. , on , No. , on , No. , on , No. , respectively.

Respectfully submitted,
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APPLICATION FOR

UNITED STATES LETTERS PATENT

Truncated Glucanase with Enhanced Activity and Method for Making the same

Inventor:

Lie-Fen Shyur
Jui-Lin Chen
Ning-Sun Yang

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a truncated form of 1,3-1,4- -D-Glucanase (lichenase, EC 3.2.1.73) with enhanced enzymatic activity and thermal tolerance.

Description of the Related Art

1,3-1,4-β-D-Glucanase is an endo-β-D-glucanase that can specifically hydrolyze 1,4-β-D-glucosidic bonds adjacent to 1,3-β-linkages in mix-linked β-glucans, yielding mainly cellobiosyltriose and cellotriosyltetraose. The enzyme has received much attention in both basic and applied research areas because of its enzymatic functions and importance in industrial applications. Supplementation of this fibrolytic enzyme in animal feed is one of the approaches for increasing the feed conversion efficiency and growth-rate of non-ruminal animals (Bedford et al., 1992; Selinger et al., 1996). This enzyme is also attractive for its application in the brewing industry. This enzyme has been used to substitute or supplement malt enzymes for reducing the industrial processing problem(s) caused by β-glucans from cell walls of the starchy seed endosperm, which include, for example, the reduced yield of extract, lowered rates of wort separation and beer filtration, formation of hazes and gelatinous precipitates in beer (Uhilg, 1998). However, the wide use of 1,3-1,4-β-D-glucanase as an industrial enzyme in general has a major drawback, that is, the limitation imposed by the thermal stability of the enzyme during industrial processes. For

instance, the elevated temperatures employed in the malting process (50-70 °C) or the feed-pelleting and/or expansion processes (65-90 °C) may cause severe inactivation of the enzyme. Therefore, creation of heat-resistant enzymes would overcome the aforementioned problem. Moreover, an enzyme with high catalytic activity would be more desirable in industrial applications in terms of cost-effectiveness.

Fibrobacter succinogenes is a microorganism that plays a major role in plant fiber digestion in the rumen. From this organism, several enzymes related to the digestion of cellulose or hemicellulose polymers of plant cell wall have been isolated and studied (Selinger et al., 1996). One of the *Fibrobacter succinogenes* emzymes, 1,3-1,4-β-D-glucanase or Fsβ-glucanase, is isolated and characterized by Teather et al. (1988 & 1990). This enzyme consists of a protein sequence with circular permutation in which two highly conservative catalytic domains of the enzyme are in a reverse orientation, as compared to that of 1,3-1,4-β-D- glucanases from other sources (Teather & Erfle, 1990; Schimming et al., 1992; Heinemann & Hahn, 1995). Five repeated serine-rich regions are found in the C-terminal, which are nonhomologous relative to bacilli or other bacterial 1,3-1,4-β-D-glucanases.

One objective of the present invention is to provide a new form of glucanase having both enhanced enzymatic activity and improved thermal stability. This objective is achieved by truncating a wild-type 1,3-1,4-β-D- glucanase whereby producing a shortened form of the enzyme. This truncated form of glucanase, with

significant enhancement both in the enzymatic activity and in the thermal stability, and the method for producing the truncated enzyme are hereby disclosed.

SUMMARY OF THE INVENTION

According to the present invention, both the enzymatic activity and the thermal stability of the *Fibrobacter succinogenes* glucanase enzyme can be greatly enhanced by removing a number of amino acid residues from its C-terminal. According to another aspect of the invention, the removal of the C-terminal portion of the enzyme can be achieved either by modification of the gene encoding for the enzyme prior to protein expression or by post-expression modification of the enzyme at the protein level. For example, in a preferred embodiment, pre-expression modification is carried out using a PCR-based gene truncation method, resulting in a truncation of the wild-type enzyme for glucanase. The truncated gene is then incorporated into an expression vector and expressed in E.coli, producing a truncated glucanase (hereinafter referred as "PCR-TF-glucanase") that is about 10 kDa smaller than the full-length wild-type enzyme. In another preferred embodiment, truncated glucanase can also be made by first expressing the wild-type enzyme and then incubating the wild type enzyme for a certain period of time and under certain conditions. The resulting truncated enzyme, with a molecular weight of approximately 27.72 kDa, is hereinafter referred as "TF-glucanase".

Both truncated enzymes, i.e., TF-glucanase, which directly derived from the full-length protein, and PCR-TF-glucanase, which is expressed from a PCR-truncated gene, show an approximate 3.9-fold increase in the specific activity as compared to that of the full-length enzyme. The specific activity, measured with lichenan as the substrate, for the wild-type, TF-glucanase, and PCR-TF-glucanase enzymes is 2065 , 7980 , and 7833 μmol/min/mg, respectively. The specific activity, if measured with barley β-glucan as substrate, is 2600, 7682, and 7975 μmol/min/mg, repectively. For comparison, the specific activity of a hybrid 1,3-1,4-β-D-glucanase, H(A16-M), constructed from the *Bacillus macerans* and *Bacillus amyloliquefaciens* enzymes (Politz, et al., 1993), is reportedly 4,890 μmol/min/mg with lichenan as the substrate at 65 °C (Hahn et al., 1994). The V_{max} value for a fungal 1,3-1,4-β-D-glucanase from *Orpinomyces* strain PC-2 with lichenan and barley β-glucan as the substrate at pH 6.0 and 40 °C are 3,790 μmol/min/mg and 5,320 μmol/min/mg, respectively (Chen et al., 1997). A commercially available 1,3-1,4-β-D-glucanase (lichenase, Megazyme International Ireland Ltd.) produced from *Bacillus subtilis* has a specific activity of 118 U/mg. Therefore, the truncated enzymes of the present invention are 1.5-, 1.6- and 68-fold higher in specific activity than the *Orpinomyces* 1,3-1,4-β-D-glucanase, H(A16-M) 1,3-1,4-β-D-glucanase and the Bacillus lichenase (Megazyme). The PCR-TF-glucanase and TF-glucanase, to applicants' knowledge, is the most active 1,3-1,4-β-D-glucanase.

In addition to the enhanced specific activity, the truncated glucanse has an improved thermal stability. For example, TF-glucanase and PCR-TF-glucanase produced in preferred embodiments, can retain 80-85 % of their original enzymatic activity after a 10 minute incubation at 90 °C, whereas the full-length enzyme can retain only 30 % of its original enzymatic activity after the same heat treatment. As a further comparison, the Bacillus lichenase from Megazyme company retains less than 10% of its activity under the same conditions. Under more severe conditions, such as being boiled for 10-30 minutes, PCR-TF-glucanase still retains 55-70% of its original activity.

The various features of novelty which characterize the invention are pointed out with particularity in the claims annexed to and forming a part of the disclosure. For a better understanding of the invention, its operating advantages, and specific objects attained by its use, reference should be had to the drawing and descriptive matter in which there are illustrated and described preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings, like reference characters denote similar elements throughout the several views:

FIG. 1 is a genetic map of pJI10 plasmid deduced from the prior art references, which contains a full-length genetic code for 1,3-1,4-β-D-glucanase of *Fibrobacter succinogenes*.

FIG. 2 is the amino acid sequence of TF-glucanase (SEQ ID NO: 1) as one embodiment of present invention, and its corresponding genetic nucleotide sequence (SEQ ID NO: 4).

FIG. 3 is the amino acid sequence of PCR-TF-glucanase (SEQ ID NO: 2)as another embodiment of present invention, and its corresponding genetic nucleotide sequence (SEQ ID NO: 5).

FIG. 4 is the kinetic data showing the truncated enzyme of the present invention has an enhanced enzymatic properties over the wild-type enzyme and a currently available commercial enzyme.

FIG. 5 is the data showing the truncated enzyme of the present invention possess a great thermal stability.

FIG. 6 is the amino acid sequence of the wild-type glucanase (SEQ ID NO: 3) as described by Teather and Erfle, and its corresponding genetic nucleotide sequence (SEQ ID NO: 6).

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

Example I: Enzyme Truncation at the DNA Level.

Subcloning of Wild-type Glucanase Gene

pJI10, a plasmid containing a wild-type gene of *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase (alse known as Fsβ-glucanase), can be used as a template for the purpose of subcloning the gene, although other DNA templates can also be satisfactorily used as long as they contain the desired glucanase gene. pJI10, whose genetic map is delineated in FIG. 1, is fully described in the prior art by Teather and Erfle in "Cloning and expression of a *Bacteriod succinogenes* mixed-linkage β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase) gene in *Escherichia coli*", *Applied and Environmental Microbiology*, 54:2672-2676 (1988) and "DNA sequence of a *Fibrobacter succinogenes* mixed-linkage β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase) gene", *J. Bacteriology*, 172:3837-3841 (1990). Thus, it is believed unnecessary to further describe the process of cloning the glucanase gene herein.

The full-length cDNA of Fsβ-glucanase in a DNA template, such as the pJI10 plasmid as used in the preferred embodiment described herein, is amplified and introduced with a *Nco I* and an *EcoR I* restriction enzyme recognition sites at 5' and 3' ends, respectively, by using a PCR-based method. The two primers designed for introducing the *Nco I* and *Eco RI* sites are 5'TCACCACCATGGTTAGCGCAAAG-

3', and 5'GCCACGAATTCTGTTCAAAGTTC AC-3', respectively. The PCR reaction is performed with a thermo-cycling program as follows: (94 °C, 5 min; 55 °C, 1 min, 72 °C, 1 min for 1 cycle), (94 °C, 1 min; 55 °C, 1 min, 72 °C, 1 min for 30 cycles), (94 °C, 1.5 min; 55 °C, 1.5 min, 72 °C, 10 min for 1 cycle). The resulting amplified DNA fragments are digested with *Nco I* and *Eco RI*, purified, and ligated onto the pET26b (+) vector which is pre-digested with *Nco I* and *Eco RI*. The sequence of Fsβ-glucanase can be confirmed by any conventional DNA sequencing methods, such as the chain termination method (Sanger, 1977). In this DNA construct, a *pel* B leading peptide at the N-terminus and extra 19 amino acid residues including 6X-histidine tag at the C-terminus to facilitate protein purification are included. The recombinant plasmid encoding for the wild-type enzyme is then transformed into *E. coli* BL21 (DE3) host.

It is to be understood that the purpose of practice of the present invention, the wild-type glucanase may be obtained from sources other than pJI10 originated by Teather and Erfle (1988 & 1990). The wild-type gene from such other sources may vary in sequence in certain regions non-critical to the enzyme's function. Similarly, the truncated glucanase of the present invention may have varied sequences in regions not critical to the enzyme's function. Therefore, the present invention is not limited to the exact sequence as disclosed herein. As used in the claims annexed to this disclosure, the phrases "substantially identical" and "substantially corresponding" mean that the claims cover enzymes or DNA coding fragments that have minor

sequence variations from the specified sequences that do not affect the enzyme's enzymatic functions to any significant degrees.

Gene truncation

The gene for 1,3-1,4-β-D-glucanase (PCR-TF-glucanase) can be truncated by using a PCR method, which uses Oligo A and Oligo B as a pair of specific primers and the full length cDNA of Fsβ-glucanase in pJI10 as template. Oligo A: 5'-CAGCCGGCGATGGCCATGGTTAGCGCA-3' and oligo B: 5'-CTGCTAGAAGAATTCGGAGCAGGTTCGTC-3', are designed to amplify both strands of the gene corresponding to the amino acid sequence from methionine 1 to proline 248. The amplified DNA fragments are digested with *Nco I* and *Eco RI* and then ligated with a pET26b (+) vector (purchased from Novagen, WI, USA) which is pre-digested with *Nco I* and *Eco RI*, forming a recombinant plasmid containing a truncated Fsβ-glucanase gene. The truncated gene of Fsβ-glucanase in the recombinant plasmid can be confirmed by a chain termination DNA sequencing method (Sanger, 1977). In this DNA construct, a *pel* B leading peptide at the N-terminus and an extra 19 amino acid residues with a 6X-histidine tag at the C-terminus with respect to that of TF-glucanase sequence are included. Finally, the plasmid containing the truncated glucanase gene can then be transformed into *E. coli* BL21(DE3) host, purchased from Novagen, WI, USA. Of course, other gene truncation methods or agents may be used satisfactorily.

In general, any method may be used if it can transfer or place a desired portion of Fsβ-glucanase gene in between an initiation codon and a stop codon of a expression frame of a suitable vector may be used.

Expression of Recombinant Glucanase Genes in E. Coli.

5 ml of pre-grown culture of the BL21 (DE3) bacterial strain carrying the pET26b (+) plasmid containing the Fsβ-glucanase gene or a truncated variation is added to 500 ml of fresh LB broth containing 30 μg/ml kanamycin. The culture is shaken vigorously at 33 °C until the OD reading at 600 nm reaches 0.4-0.6. Then, add 1 mM of IPTG to the culture and further incubate for 16 hour at 33 °C. Under those culture conditions, the wild-type glucanase or truncated enzyme can be effectively expressed and secreted into the medium as a soluble protein. In general, same culture conditions can be used for expression of either full-length wild-type gene or a truncated gene. Of course, other variations of culture conditions are possible and the above stated parameters are merely provided as an example. For instance, the LB culture medium can be replaced with NZCYM or M9 medium and the incubation temperature can be varied within a wide range.

LB (pH7.0) contains: 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, and 1%(w/v) NaCl. NZCYM medium (pH 7.0) contains: 1% (w/v) NZ amine (Sigma Chemical Co., MO, USA), 0.5% (w/v) NaCl, 0.5% (w/v) bacto-yeast extract, 0.1% (w/v) casamino acids, and 0.2% (w/v) MgSO4(7H2O). M9 contains: 1x

M9 salt [1.28% (w/v) Na2HPO4(7 H2O), 0.3% (w/v) KH2PO4, 0.05% (w/v) NaCl, 0.1% (w/v) NH4Cl], and 0.4% glucose.

Purification of glucanases in the culture medium.

The wild-type or truncated forms of 1,3-1,4-β-D-glucanases produced in the above-described procedure can be further purified. The culture at the end of the planned incubation is centrifuged at 8,000 x g for 15 min at 4 °C. The supernatant, containing approximately 80-85% of the expressed protein product, is collected and concentrated ten (10) times using a Pellicon Cassette concentrator (Millipore, Bedford, MA) with a 10,000 M_r cut-off membrane. The concentrated culture supernatant is then dialyzed against 50 mM Tris-HCl buffer, pH 7.8 (buffer A) and loaded onto a Sepharose Q FF (Pharmacia, Sweden) column pre-equilibrated with the same buffer. 1,3-1,4-β-D-Glucanase proteins, either the wild-type or a truncated form of the enzyme, is collected from the eluants of the column eluted with a 0-1 M NaCl salt gradient in buffer A. A second Ni-NTA affinity column equilibrated with 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, and 10 mM imidazole buffer (buffer B) is then employed for further purification of the enzymes. From a 10-300 mM imidazole gradient eluant, homogeneous enzyme preparation can be obtained, as verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Protein concentration is quantified as described by Bradford (1976) with bovine serum albumin (BSA) as the standard. It is to be understood, however, that the purification method forms no part of the invention, other purification

techniques, either existing now or to be developed in future, can be satisfactorily used.

Example II: Enzyme Truncation at the Protein Level.

The first step in truncating the glucanase at the protein level is to produce the full-length wild type enzyme, which is fully described in the preceding sections in Example I. In summary, *E. coli* host cells harboring the full length Fsβ-glucanase gene are cultured in either LB, NZCYM, or M9 medium under conditions suitable for cell growth and protein expression. (IPTG may be added at a certain point of the incubation to induce the gene transcription). The active enzyme in the culture medium is then collected (by centrifugation and recovering the supernatant) and concentrated to an appropriate volume on a Pellicon Cassette concentrator (Millipore, USA) with a 10,000 M_r cut-off membrane. The enzyme in the supernatant accounts about 80-85% of total expressed enzyme, and the remaining 15-20% is found in the cell pellet.

After concentrating the culture supernatant, the second step is to conduct post-expression modification of the protein structure. As an example, such post-expression modification to produce shortened protein can be achieved by incubating the concentrated culture supernatant for a prolonged period of time under certain conditions. In a preferred embodiment, the supernatant is incubated for 10-14 days at a temperature within the range from 4 °C to 37 °C. Although, it is possible to use the same prolonged incubation process to obtain the truncated enzyme from the

cell pellet which contains about 15-20% of the total enzyme, it is a less efficient method. Other incubation conditions, however, may also produce satisfactory results. It is believed that a propyl endopeptidase known as protease II in *E. coli* (Kanatani et al., 1991) may play a major proteolytic role in the generation of TF-glucanase from the full-length enzyme.

Confirmation of the Improved Enzymatic Property

The following description imposes no limitations to the present invention, but merely serves to further characterize the above disclosed embodiments.

Structural Characterisation of Truncated Enzymes

The post-expression truncation of the wild-type enzyme produces a mixture of truncated enzyme molecules with a molecular weight ranging from 27 kDa to 37.5 kDa. However, there is a dominant species, referred as TF-glucanase, that has a molecular weight of approximately 27.7 kDa, that is, about 10 kDa smaller than that of the full-length enzyme. This dominant TF-glucanase is formed after 10 to 14 days post-expression incubation in the LB medium at 25 °C, and is stable and active even if when the incubation time is extended for up to 45 days at 25 °C. The sequence of this dominant TF-glucanase is presented in FIG 2, which suggests that TF-glucanase is produced when approximately 80 amino acid residues are removed from the C-terminus of the wild-type enzyme. On the other hand, the PCR-generated truncated enzyme, i.e., PCR-TF-glucanase, has a molecular weight of 29.7 kDa, and shares the

same amino acid sequence with TF-glucanase except that PCR-TF-glucanase has 19 extra amino acid residues at the C-terminus, see FIG 3. Five P-X-S-S-S-S repeats located in the C-terminal portion of the wild-type enzyme are absent from either TF-glucanase or PCR-TF-glucanase. The symbol P represents poline, S presents serine, and X represents an uncharged residue, such as Alanine, Proline, or Glutamine.

Biochemical and Kinetic Characterization

Zymogram is used to measure enzymatic activity of various forms of glucanase, which is performed essentially according to a reported method (Piruzian et al.,1998). A 12% SDS polyacrylamide gel containing lichenan (1 mg/ml) and protein samples in sample buffer (Laemmli, 1970) pretreated at 90 °C for 10 min are prepared for the zymogram analysis. After electrophoresis, the gel is rinsed twice with 20% isopropanol in 50 mM sodium citrate buffer (pH 6.0) for 20 min to remove SDS, and then equilibrated in 50 mM sodium citrate buffer for 20 min. Before staining with Congo red solution (0.5 mg/ml), the gel is pre-incubated at 40 °C for 10 min. The protein bands with 1,3-1,4-β-D-glucanase activity can be visualized using the Congo red staining.

With reference to FIG. 4, experiments on kinetic studies are mainly performed using lichenan as the substrate. The specific activity of the wild-type (full length) Fsβ-glucanase, TF-glucanase, and PCR-TF-glucanase enzymes are 2065 ± 82, 7980 ± 341, and 7833 ± 334 U/mg, respectively. Thus, a 3.9-fold increase in the specific activity is achieved in the truncated enzymes as compared with the wild-type enzyme.

A slight decrease (1.5-fold) in the affinity for lichenan (K_m) is detected in the TF-glucanase and PCR-TF-glucanase relative to the wild-type enzyme. The turnover number (k_{cat}) and catalysis efficiency (k_{cat}/K_m) are 2 or 3-fold higher in the truncated enzymes than in the wild-type enzyme. The TF-glucanase is shown to have similar kinetic properties to those of PCR-TF-glucanase. The kinetic properties of the three forms of glucanase with barley β-glucan as substrate is also examined. The V_{max} and K_m values for wild-type, TF-glucanase, and PCR-TF-glucanase enzymes with barley β-glucan as substrate are 2643 ± 77, 7682 ± 38, and 7975 ± 22 μmol/min/mg, and 2.93 ± 0.18, 3.05 ± 0.03, and 3.86 ± 0.02 mg/ml, respectively. For wild-type and truncated enzymes, the optimum temperature is around 50 °C when assayed with 50 mM citrate buffer (pH 6.0). Truncated and wild-type enzymes also show a similar pH profile within the range from 4 to 10 with the optimum pH ranging from 6 to 8.

Recovery of enzymatic activity following a 10 minutes treatment at 90 °C is examined, see FIG. 5. The wild-type enzyme and PCR-TF-glucanase show 8% and 40%, respectively, of their original activity 3 minutes after transferring the heat-treated enzymes to room temperature. However, PCR-TF-glucanase recovers more than 80% of its original activity after be transferred to a 25 °C environment for 12 minutes while the recovery is 27% for the wild-type enzyme. Furthermore, the restored enzymatic activity of the wild-type enzyme is not stable and decreases to less than 10% of its original activity when incubated at 25 °C for four hours. In contrast, both PCR-TF-glucanase and TF-glucanase can maintain 70% of their original activity

when incubated at 25 °C for 24 hour. PCR-TF-glucanase can recover 55 to 70 % of its original activity after being boiled at about 100 °C for 10-30 minutes.

Lastly, the catalytic properties of a commercially available Bacillus lichenase from Megazyme Company are examined as a comparison to the truncated enzyme made according the present invention. The turnover rate (k_{cat}) of lichenase (Megazyme) is 47 s^{-1} when using barley β-glucan as the substrate, and is 33 s^{-1} with lichenan as the substrate, representing values 85-fold and 118-fold lower than those of PCR-TF-glucanase with respective substrates. Although the lichenase (Megazyme) has a higher optimum temperature (5-10 °C higher) than PCR-TF-glucanase, it is much less thermal stable. Enzymatic activity of the lichenase (Megazyme) can restored less than 10 % of its original activity following a 90 °C heat treatment for 10 minute, whereas PCR-TF-glucanase, as mentioned above, readily recovers 80-85 % of its original activity.

While there have been shown, described and pointed out the features of the invention as applied to a preferred embodiment thereof, it will be understood that various omissions and substitutions and changes in the form and details of the devices illustrated, and in their operation, may be made by those skilled in the art without departing from the spirit of the invention. For example, it is expressly intended that all combinations of those elements and/or method steps which perform substantially the same function in substantially the same way to achieve the same results are within the scope of the invention.

The following list provides the sources of the references mentioned in the disclosure which may be helpful to people in the art to practice the present invention. The contents of all the listed publications are expressly incorporated into the disclosure by reference.

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CLAIMS

We claim:

1. A truncated glucanase having an amino acid sequence with a total number of amino acid residues between 200 and 321, at least 200 of said amino acid residues forming a linear sequence substantially identical to a portion of the amino acid sequence of a wild-type glucanase from *Fibrobacter succinogenes*.

2. The truncated form of glucanase of claim 1, wherein said linear sequence contains no PXSSSS repeats.

3. The truncated form of glucanase of claim 1, wherein said amino acid sequence of a wild-type glucanase is identical to SEQ ID NO: 3 in FIG 6.

4. The truncated form of glucanase of claim 3, wherein said portion of the amino acid sequence starts from residue 25 of SEQ ID NO: 3 in FIG. 6 and extends towards the C-terminal of SEQ ID NO:3, covering less than 321 amino acid residues.

5. The truncated form of glucanase of claim 4, wherein said portion of the amino acid sequence covers more than 246 amino acid residues.

6. The truncated form of glucanase of claim 1, having an amino acid sequence substantially identical to SEQ ID NO:1 in FIG 2.

7. The truncated form of glucanase of claim 1, having an amino acid sequence substantially identical to SEQ ID NO:2 in FIG 3.

8. A DNA fragment having an initiation codon, a stop codon and a coding sequence between said two codons, said coding sequence substantially corresponding to said amino acid sequence of claim 1.

9. The DNA fragment of claim 8, wherein said DNA fragment has a sequence of nucleotide residues substantially identical to SEQ ID NO: 4 in FIG 2.

10. The DNA fragment of claim 8, wherein said DNA fragment has a sequence of nucleotide residues substantially identical to SEQ ID NO: 5 in FIG 3.

11. A method of producing said truncated glucanase of claim 1, comprising:

(a) growing in a culture medium a bacterial strain carrying a plasmid containing a gene encoding for a wild-type 1,3-1,4-β-D-glucanase from *Fibrobacter succinogenes*,

(b) adding to said culture medium an inducer to induce expression of said gene and continuing said growing of step (a),

(c) centrifuging said culture medium to produce a supernatant,

(d) incubating said supernatant to produce said truncated glucanase, and

(e) collecting and purifying said truncated glucanase from said supernatant.

12. The method of claim 11, wherein said supernatant in step (d) is incubated for at least 7 days at 4 °C or a higher temperature.

13. The method of claim 11, wherein said supernatant in step (d) is incubated for a period ranging from 10 days to 14 days and at a temperature ranging from 4 °C to 37 °C.

14 The method of claim 11, wherein said supernatant in step (d) is incubated for 14 days at 37 °C.

15. A method of producing said truncated glucanase of claim 1, comprising:

(a) amplifying a DNA fragment using a PCR method from a DNA template containing a gene encoding for a wild-type glucanase from *Fibrobacter succinogenes*, said DNA fragment substantially corresponding to a portion of said gene,

(b) subcloning said amplified DNA fragment in an expression vector,

(c) transferring said expression vector harbouring said DNA fragment into a bacterial strain,

(d) growing said bacterial strain in a culture medium for a period of time and inducing expression of said DNA fragment, with or without adding an inducer, to produce a sufficient amount of protein products, and

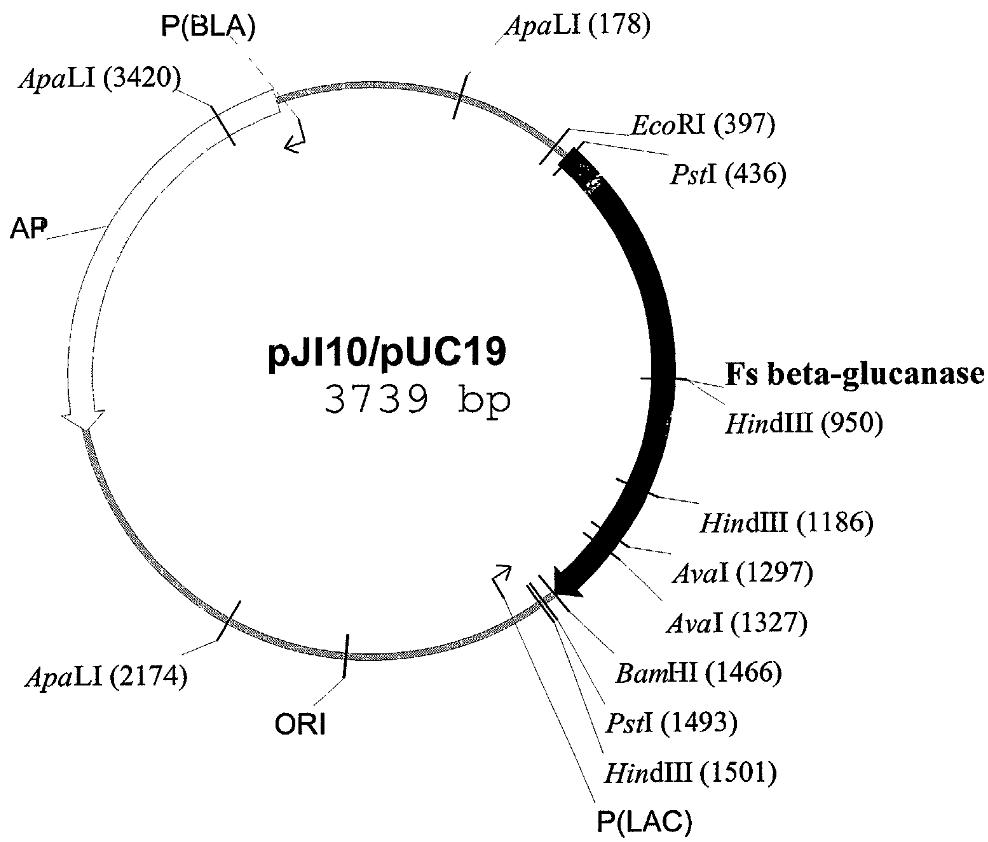
(e) collecting and purifying protein expression products from said culture medium.

16. The method of claim 15, wherein said DNA fragment amplified in step (a) has a sequence substantially identical to SEQ ID NO: 6 in FIG. 6.

ABSTRACT

A truncated glucanase with an improved thermal stability and a higher specific enzymatic activity than the wild-type enzyme. The truncated glucanase is obtained by removing a number of amino acid residues from the C-terminal of the wild-type 1,3-1,4-β-D-glucanase of *Fibrobacter succinogenes*. The removal of the C-terminal amino acid residues can be conducted at the genetic level by modifying the gene encoding for the wild type enzyme using, for example, a PCR-based method. Or, it can also be conducted at the protein level by first producing the wild-type enzyme protein and then subjecting the wild-type protein to certain protease action to remove a portion of its C-terminal.

Fig. 1



09654652 .090500

Fig. 2 The amino acid sequence in Fig. 2 is SEQ ID NO: 1
the DNA sequence in Fig. 2 is SEQ ID NO: 4

```
ATGGTTAGCGCAAAGGATTTTAGCGGTGCCGAACTCTACACGTTAGAAGAAGTTCAGTAC
 M  V  S  A  K  D  F  S  G  A  E  L  Y  T  L  E  E  V  Q  Y     20

GGTAAGTTTGAAGCCCGTATGAAGATGGCAGCCGCATCGGGAACAGTCAGTTCCATGTTC
 G  K  F  E  A  R  M  K  M  A  A  A  S  G  T  V  S  S  M  F     40

CTCTACCAGAATGGTTCCGAAATCGCCGATGGAAGGCCCTGGGTAGAAGTGGATATTGAA
 L  Y  Q  N  G  S  E  I  A  D  G  R  P  W  V  E  V  D  I  E     60

GTTCTCGGCAAGAATCCGGGCAGTTTCCAGTCCAACATCATTACCGGTAAGGCCGGCGCA
 V  L  G  K  N  P  G  S  F  Q  S  N  I  I  T  G  K  A  G  A     80

CAAAAGACTAGCGAAAAGCACCATGCTGTTAGCCCCGCCGCCGATCAGGCTTTCCACACC
 Q  K  T  S  E  K  H  H  A  V  S  P  A  A  D  Q  A  F  H  T    100

TACGGTCTCGAATGGACTCCGAATTACGTCCGCTGGACTGTTGACGGTCAGGAAGTCCGC
 Y  G  L  E  W  T  P  N  Y  V  R  W  T  V  D  G  Q  E  V  R    120

AAGACGGAAGGTGGCCAGGTTTCCAACTTGACAGGTACACAGGGACTCCGTTTTAACCTT
 K  T  E  G  G  Q  V  S  N  L  T  G  T  Q  G  L  R  F  N  L    140

TGGTCGTCTGAGAGTGCGGCTTGGGTTGGCCAGTTCGATGAATCAAAGCTTCCGCTTTTC
 W  S  S  E  S  A  A  W  V  G  Q  F  D  E  S  K  L  P  L  F    160

CAGTTCATCAACTGGGTCAAGGTTTATAAGTATACGCCGGGCCAGGGCGAAGGCGGCAGC
 Q  F  I  N  W  V  K  V  Y  K  Y  T  P  G  Q  G  E  G  G  S    180

GACTTTACGCTTGACTGGACCGACAATTTTGACACGTTTGATGGCTCCCGCTGGGGCAAG
 D  F  T  L  D  W  T  D  N  F  D  T  F  D  G  S  R  W  G  K    200

GGTGACTGGACATTTGACGGTAACCGTGTCGACCTCACCGACAAGAACATCTACTCCAGA
 G  D  W  T  F  D  G  N  R  V  D  L  T  D  K  N  I  Y  S  R    220

GATGGCATGTTGATCCTCGCCCTCACCCGCAAAGGTCAGGAAAGCTTCAACGGCCAGGTT
 D  G  M  L  I  L  A  L  T  R  K  G  Q  E  S  F  N  G  Q  V    240

CCGAGAGATGACGAACCTGCTCCG
 P  R  D  D  E  P  A  P                                        248
```

Fig. 3
Shyur et al

Fig. 3 The amino acid sequence in Fig. 3 is SEQ ID NO: 2

The DNA sequence in Fig. 3 is SEQ ID NO: 5

```
ATGGTTAGCGCAAAGGATTTTAGCGGTGCCGAACTCTACACGTTAGAAGAAGTTCAGTAC
 M  V  S  A  K  D  F  S  G  A  E  L  Y  T  L  E  E  V  Q  Y    20

GGTAAGTTTGAAGCCCGTATGAAGATGGCAGCCGCATCGGGAACAGTCAGTTCCATGTTC
 G  K  F  E  A  R  M  K  M  A  A  A  S  G  T  V  S  S  M  F    40

CTCTACCAGAATGGTTCCGAAATCGCCGATGGAAGGCCCTGGGTAGAAGTGGATATTGAA
 L  Y  Q  N  G  S  E  I  A  D  G  R  P  W  V  E  V  D  I  E    60

GTTCTCGGCAAGAATCCGGGCAGTTTCCAGTCCAACATCATTACCGGTAAGGCCGGCGCA
 V  L  G  K  N  P  G  S  F  Q  S  N  I  I  T  G  K  A  G  A    80

CAAAAGACTAGCGAAAAGCACCATGCTGTTAGCCCCGCCGCCGATCAGGCTTTCCACACC
 Q  K  T  S  E  K  H  H  A  V  S  P  A  A  D  Q  A  F  H  T   100

TACGGTCTCGAATGGACTCCGAATTACGTCCGCTGGACTGTTGACGGTCAGGAAGTCCGC
 Y  G  L  E  W  T  P  N  Y  V  R  W  T  V  D  G  Q  E  V  R   120

AAGACGGAAGGTGGCCAGGTTTCCAACTTGACAGGTACACAGGGACTCCGTTTTAACCTT
 K  T  E  G  G  Q  V  S  N  L  T  G  T  Q  G  L  R  F  N  L   140

TGGTCGTCTGAGAGTGCGGCTTGGGTTGGCCAGTTCGATGAATCAAAGCTTCCGCTTTTC
 W  S  S  E  S  A  A  W  V  G  Q  F  D  E  S  K  L  P  L  F   160

CAGTTCATCAACTGGGTCAAGGTTTATAAGTATACGCCGGGCCAGGGCGAAGGCGGCAGC
 Q  F  I  N  W  V  K  V  Y  K  Y  T  P  G  Q  G  E  G  G  S   180

GACTTTACGCTTGACTGGACCGACAATTTTGACACGTTTGATGGCTCCCGCTGGGGCAAG
 D  F  T  L  D  W  T  D  N  F  D  T  F  D  G  S  R  W  G  K   200

GGTGACTGGACATTTGACGGTAACCGTGTCGACCTCACCGACAAGAACATCTACTCCAGA
 G  D  W  T  F  D  G  N  R  V  D  L  T  D  K  N  I  Y  S  R   220

GATGGCATGTTGATCCTCGCCCTCACCCGCAAAGGTCAGGAAAGCTTCAACGGCCAGGTT
 D  G  M  L  I  L  A  L  T  R  K  G  Q  E  S  F  N  G  Q  V   240

CCGAGAGATGACGAACCTGCTCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTC
 P  R  D  D  E  P  A  P  N  S  S  S  V  D  K  L  A  A  A  L   260

GAGCACCACCACCACCACCACTGA
 E  H  H  H  H  H  H  *                                       267
```

Fig. 4.

Table 1. Comparison of kinetic properties of *F. succinogenes* and *B. subtilis* 1,3-1,4-β-D-glucanases

Enzyme	Specific activity (U/mg)	k_{cat} ($^{-1}$s)	Opt. Temperature (°C)	Opt. pH
Wild-type	2065 ± 82	1296 ± 51	50 (at pH 6.0)	6.0-8.0
TG-Glucanase	7980 ± 341	3695 ± 158	50 (at pH 6.0)	6.0-8.0
PCR-TF-Glucanase	7833 ± 334	3911 ± 166	50 (at pH 6.0)	6.0-8.0
Lichanase (Megazyme)	118[a]	47.2[a]	60 (at pH 6.5)[a]	6.5-7.0[a]
	82.6 ± 0.96	33.0 ± 0.38	55 (at pH 7.0)	

The kinetics was performed with lichenan (6mg/mL) as substrate in 50 mM citrate buffer (pH 6.0) or in 50mM phosphate buffer (pH 7.0), and at optimum temperature as indicated.

[a]: Data was taken from *Megazyme* instruction brochure of lichenase. The kinetics was done with barley β-glucan (5mg/mL) as substrate.

Fig. 5

Table 3. Reactivation of PCR-TF-glucanase at 25 °C after heat treatment

Heat treatment	Recovery time (min)	Relative activity (%)
90 °C, 10 min	10	68
	20	81
90 °C, 30 min	10	61
	20	67
100 °C, 10 min	10	68
	20	72
100 °C, 30 min	10	55
	20	56

Fig.6a The amino acid sequence in Fig. 6 is SEQ ID NO: 3

(part a) The DNA sequence in Fig. 6 is SEQ ID ~~ID~~ NO: 6

```
ATGAACATCAAGAAAACTGCAGTCAAGAGCGCTCTCGCCGTAGCAGCCGCAGCAGCAGCC
 M  N  I  K  K  T  A  V  K  S  A  L  A  V  A  A  A  A  A  A   20

CTCACCACCAATGTTAGCGCAAAGGATTTTAGCGGTGCCGAACTCTACACGTTAGAAGAA
 L  T  T  N  V  S  A  K  D  F  S  G  A  E  L  Y  T  L  E  E   40

GTTCAGTACGGTAAGTTTGAAGCCCGTATGAAGATGGCAGCCGCATCGGGAACAGTCAGT
 V  Q  Y  G  K  F  E  A  R  M  K  M  A  A  A  S  G  T  V  S   60

TCCATGTTCCTCTACCAGAATGGTTCCGAAATCGCCGATGGAAGGCCCTGGGTAGAAGTG
 S  M  F  L  Y  Q  N  G  S  E  I  A  D  G  R  P  W  V  E  V   80

GATATTGAAGTTCTCGGCAAGAATCCGGGCAGTTTCCAGTCCAACATCATTACCGGTAAG
 D  I  E  V  L  G  K  N  P  G  S  F  Q  S  N  I  I  T  G  K  100

GCCGGCGCACAAAAGACTAGCGAAAAGCACCATGCTGTTAGCCCCGCCGCCGATCAGGCT
 A  G  A  Q  K  T  S  E  K  H  H  A  V  S  P  A  A  D  Q  A  120

TTCCACACCTACGGTCTCGAATGGACTCCGAATTACGTCCGCTGGACTGTTGACGGTCAG
 F  H  T  Y  G  L  E  W  T  P  N  Y  V  R  W  T  V  D  G  Q  140

GAAGTCCGCAAGACGGAAGGTGGCCAGGTTTCCAACTTGACAGGTACACAGGGACTCCGT
 E  V  R  K  T  E  G  G  Q  V  S  N  L  T  G  T  Q  G  L  R  160

TTTAACCTTTGGTCGTCTGAGAGTGCGGCTTGGGTTGGCCAGTTCGATGAATCAAAGCTT
 F  N  L  W  S  S  E  S  A  A  W  V  G  Q  F  D  E  S  K  L  180

CCGCTTTTCCAGTTCATCAACTGGGTCAAGGTTTATAAGTATACGCCGGGCCAGGGCGAA
 P  L  F  Q  F  I  N  W  V  K  V  Y  K  Y  T  P  G  Q  G  E  200

GGCGGCAGCGACTTTACGCTTGACTGGACCGACAATTTTGACACGTTTGATGGCTCCCGC
 G  G  S  D  F  T  L  D  W  T  D  N  F  D  T  F  D  G  S  R  220

TGGGGCAAGGGTGACTGGACATTTGACGGTAACCGTGTCGACCTCACCGACAAGAACATC
 W  G  K  G  D  W  T  F  D  G  N  R  V  D  L  T  D  K  N  I  240

TACTCCAGAGATGGCATGTTGATCCTCGCCCTCACCCGCAAAGGTCAGGAAAGCTTCAAC
 Y  S  R  D  G  M  L  I  L  A  L  T  R  K  G  Q  E  S  F  N  260

GGCCAGGTTCCGAGAGATGACGAACCTGCTCCGCAATCTTCTAGCAGCGCTCCGGCATCT
 G  Q  V  P  R  D  D  E  P  A  P  Q  S  S  S  S  A  P  A  S  280

TCTAGCAGTGTTCCGGCAAGCTCCTCTAGCGTCCCTGCCTCCTCGAGCAGCGCATTTGTT
 S  S  S  V  P  A  S  S  S  S  V  P  A  S  S  S  S  A  F  V  300

CCGCCGAGCTCCTCGAGCGCCACAAACGCAATCCACGGAATGCGCACAACTCCGGCAGTT
```

Fig 6
(part b)

```
 P  P  S  S  S  S  A  T  N  A  I  H  G  M  R  T  T  P  A  V  320

GCAAAGGAACACCGCAATCTCGTGAACGCCAAGGGTGCCAAGGTGAACCCGAATGGCCAC
 A  K  E  H  R  N  L  V  N  A  K  G  A  K  V  N  P  N  G  H  340

AAGCGTTATCGCGTGAACTTTGAACACTAA
 K  R  Y  R  V  N  F  E  H  *                                349
```

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

A TRUNCATED FORM OF FIBROBACTER SUCCINOGENES 1,3-1,4-BETA-D-GLUCANASE WITH IMPROVED ENZYMATIC ACTIVITY AND THERMO-TOLERANCE

the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I also acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37 CFR 1.63(d), which occurred between the filing date of the prior application and the filing date of the continuation-in-part application, if this is a continuation-in-part application.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application: **Country:**
Appln. No.:
Filed:

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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